MYCOLOGICAL AND PATHOLOGICAL STUDIES OF *Rhytidhysterium rufulum*, A POSSIBLE AGENT OF CITRUS TWIG BLIGHT

By

JOSE LUIZ BEZERRA

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JOSE LUIZ BEZERRA

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Chairman: Dr. James W. Kimbrough
Cochairman: Dr. Norman C. Schenck
Major Department: Plant Pathology

Cultural, cytological, and developmental aspects of the patellariaceous ascomycete, *Rhytidhysterium rufulum* (Spreng.) Speg., are investigated. A comparison of the ultrastructural features of the *R. rufulum* ascus with those of other loculoascomycetes is made.

Meiosis and mitosis in the ascus and ascospores of *R. rufulum* are not significantly different to those considered typical for most ascomycetes. Ascospore delimitation begins after the ascus reaches its 16-nucleate condition. Differences in centrum development and hymenial configuration indicate that the Patellariaceae and Hysteriaceae may have to be placed in different orders.

A new species of basidiomycete parasitizing the ascocarp of *R. rufulum* is described and named *Tremella rhytidhysterii* Bezerra & Kimbr. sp. nov. The mycelial interaction between these two species is studied with both light and electron microscopy. The cytological development of basidia and basidiospores is also described. The new combination *Rhytidhysterium fuscum* (Ell. & Ev.) Bezerra & Kimbr. is proposed.

During basidial development in T. rhytidhysterii, the metabasidium becomes two-celled and two nuclei migrate through each protosterigma. One of the nuclei apparently degenerates. In culture, T. rhytidhysterii undergoes dikaryotization and dedikaryotization. Haustorial branches are formed by the mycoparasite hyphae in the presence of the host hyphae. Contact between the haustorial branches and the host hyphae was observed with both light and electron microscopy, but no penetration was observed. Tremella rhytidhysterii has a peculiar type of dolipore septum which has also been reported in Filobasidium and Filobasidiella. A possible relationship between these three genera is suggested. A unique filamentous capsular material is present in the yeast cells of T. rhytidhysterii.

The pathogenicity of R. rufulum to rough lemon (Citrus jambhiri Lush.) is investigated in living stems in moist chambers and in potted plants. Inoculation experiments conducted with R. rufulum on rough lemon plants showed that this fungus is not pathogenic to rough lemon, although it can colonize dead tissues of its host.

GENERAL INTRODUCTION

Rhytidhysterium rufulum (Spreng.) Speg. is an ascomycete with naviculate ascocarps, bitunicate asci, and dark, four-celled ascospores. It is found growing on the bark of various plants and has been considered a saprophyte by most authors. A few mycopathologists have regarded it as a weak plant pathogen. Because of its peculiar apothecioid ascocarps, this fungus has been placed in several orders of discomycetes by earlier fungal taxonomists. Modern mycologists, however, tend to assign it to the loculoascomycetes because of its bitunicate asci and mode of ascocarp development.

The presence of this fungus on species of Citrus in Florida has provided an opportunity to study anew some of the problems that are yet unresolved. These include (1) the type of centrum; (2) cytological development of excipular cells, ascogonia, ascogenous hyphae, asci, and ascospores; (3) ultrastructural aspects of ascal development and morphology; (4) cytology and ultrastructure of the host-fungus relationship; and (5) the possible pathogenic role of the fungus.

The inoperculate discomycete Mollisiella ilicincola (Berk. & Br.) Mass. (=Unguiculariopsis) has often been reported as parasitic on R. rufulum. In this study, however, yet another fungus, an undescribed species of Tremella, was found parasitic on Rhytidhysterium. A description of this new species is provided along with observations of hyphal interaction of the two fungi in culture and in mature specimens.

Various aspects of this research will be discussed in the following chapters: (I) Culture and Cytological Development of Rhytidhysterium rufulum on Citrus; (II) A New Species of Tremella Parasitic on Rhytidhysterium rufulum; and (III) Host-Fungus Relationship of Rhytidhysterium rufulum on Citrus.

CHAPTER I
CULTURE AND CYTOLOGICAL DEVELOPMENT OF *Rhytidhysterium rufulum* ON *Citrus*

Rhytidhysterium rufulum (Spreng.) Speg. is an interesting ascomycete with dark, carbonaceous, naviculate ascocarps; cylindric, bitunicate asci; and dark, four-celled ascospores. It was first described by Sprengel (1820) as *Hysterium rufulum* and cited by Saccardo (1883) as the type of his new genus *Tryblidiella*. The genus described by Saccardo was identical to *Rhytidhysterium*, an older genus proposed by Spegazzini (1881), a fact that went undetected in the literature for several years. Ellis and Everhart (1892) transferred *H. rufulum* to the genus *Tryblidium sensu* Dufour (1928), *non* Rebentish (1804), ignoring the fact that Rehm (1889) had already placed *Tryblidium* Dufour in synonymy with *Tryblidiella*. Rehm (1904) divided *Tryblidiella* into two subgenera, placing the didymosporous species into a new subgenus *Eutryblidiella* and the phragmosporous species (including *H. rufulum*) into the subgenus *Rhytidhysterium*.

Höhnel (1918), Clements and Shear (1931), Petrak (1960) and Müller and von Arx (1962) considered *Rhytidhysterium* and *Tryblidiella* as synonymous. Petrak (1960) proposed that *Rhytidhysterium* be considered the valid name because it predated *Tryblidiella* and proposed the type *R. rufulum* (Spreng.) Petrak. This new combination became superfluous because Spegazzini (1920) had already proposed the combination.

With its unique combination of characters, *Rhytidhysterium* has been placed in various orders and families including: Pezizales, family

Cenangiaceae (Rehm, 1889), and family Dermateaceae (Rehm, 1904; Clements and Shear, 1931), based on apothecial structure and shape irrespective of ascal structure; Helotiales, family Tryblidiaceae (Höhnel, 1918), based on structure and the elaborate development of the hypothecium; Phacidiales, family Hysteriaceae (Clements and Shear, 1931), based on dark, elongate apothecia opening by a cleft; Lecanorales (Nannfeldt, 1932), based on thick-walled asci, large, brown ascospores, cartilaginous to gelatinous paraphyses and iodine positive reaction in the hymenium; Hysteriales, family Patellariaceae (Luttrell, 1955, 1973; Martin, 1961; Muthappa, 1967; Kamat and Anahosur, 1973), based on structural type and development of the centrum, apothecial ascocarps, and bitunicate asci; Dothiorales, family Patellariaceae (Müller and von Arx, 1962; Gäumann, 1964; Seshadri and Muthappa, 1974), based on apothecioid ascomata, persistent paraphysoids and a persistent epithecium together with bitunicate asci; Dothideales, family Patellariaceae (von Arx and Müller, 1975), based on their 1962 conclusions. Von Arx and Müller (1975) did not believe there were sufficient characters to separate the Dothideales and Dothiorales and concluded that the Dothideales was the only acceptable order for the bitunicate ascomycetes.

It is clear from a review of the literature that most mycologists have a tendency to place Rhytidhysterium in the Patellariaceae, which would comprise all bitunicate ascomycetes with apothecial-like ascocarps having apically free paraphyses forming an epithecium. The current opinion, proposed by Luttrell (1973), advocates the placement of the Patellariaceae together with the Hysteriaceae in a separate order, namely the Hysteriales. He bases his opinion on the type of ascocarp

development, even though it is not uniform throughout the Hysteriales. In this order there are at least three structural types of internal tissues. In the first type, represented by Glonium stellatum Muhl. ex Fr. (Luttrell, 1953), a member of the Hysteriaceae, pseudoparaphyses grow downward from the roof of the cavity, forming distinct strands prior to the formation of the asci. The second type, found in R. rufulum (Muthappa, 1967; Seshadri and Muthappa, 1974; Luttrell, 1973), Tryblidiella (=Rhytidhysterium) indica Anahosur, Lecanidion caesalpini Anahosur, Tryblidaria maharashtrensis Anahosur (Kamat and Anahosur, 1973; Anahosur, 1974), and Eutryblidiella sabina (de Not.) Höhn. (Pirozynski and Reid, 1966), is characterized by paraphysoidal filaments attached at both ends, which are formed in advance of the asci and detach at their tips at maturity, branching to form an epithecioid layer. The third type corresponds to the pattern of development of Tryblidiella (=Rhytidhysterium) clavispora (Peck) Berl. & Bogl. (Muthappa, 1970), Lecanidion atratum (Hedw. ex Fr.) Endl., Tryblidaria subsidua (Cke. & Ell.) Sacc., Karschia lignyota (Fr.) Sacc. (Bellemère, 1967, 1971), and possibly Pseudoscypha abietis Reid and Pirozynski (Reid and Pirozynski, 1966; Luttrell, 1973), in which true paraphyses are formed ahead of the asci, with their tips free from the beginning. A fourth type could be represented by Melaspilea emergens (Fr.) Rehm and Baggaea pachyasca Auersw., in which paraphysoidal filaments are formed as in the first type described above, but with their apices broken at maturity (Bellemère, 1971). Therefore, the family Hysteriaceae (type 1) has a type of development quite different from that of the Patellariaceae (types 2, 3, and perhaps 4) and this fact might consequently suggest that these families

be placed in different orders, if the criterion of development is stressed. Von Arx and Müller (1975) currently propose to maintain Rhytidhysterium in the Patellariaceae largely because of its apothecioid ascocarp and bitunicate asci. However, these authors place the Patellariaceae in the suborder Dothideineae of the Dothideales near the Hysteriaceae, thus implying a close relationship. This seems difficult to justify, since the ascocarp of the Hysteriaceae in cross section is rather perithecioid. The narrow cleft of the Hysteriaceae members is not significantly different from the narrow cleft of the Lophiaceae, which is implicitly considered "perithecioid" by von Arx and Müller (1975), who place the latter group in the Pseudospaeriineae. Therefore, as in the previous instance, if the hymenial configuration is the criterion to be adopted, the families Patellariaceae and Hysteriaceae should perhaps be placed in different orders or suborders.

Muthappa (1967) studied the morphology and development of R. rufulum and recognized the following parts of the ascocarp, or discothecium as termed by Korf (1962): a) an epistroma or modified epithecium, b) a hymenium, c) a hypothecium, and d) an excipulum comprising the ectal excipulum and medullary excipulum. Bellemère (1971), in his anatomical study of the same species, adopted a more elaborate terminology which he borrowed from his earlier studies on the development of inoperculate discomycetes (Bellemère, 1967). He referred to the ascocarp as an apothecium constructed of the following: a) an epithecium; b) a hymenium with asci and primary and secondary paraphyses; c) a podium; d) a parathecial apparatus consisting of a parathecium, a parathecioid casing, and an amphithecioid lining; e) primary and secondary medullae;

and f) a conceptacle which is the remnant of the original stroma.

The centrum development in R. rufulum, briefly reported by Muthappa (1967), was more fully described by him and his colleague later (Muthappa, 1970; Seshadri and Muthappa, 1974). According to these authors, vertically arranged hyphae attached at both ends and elongating by intercalary growth appear across the upper part of the stroma prior to the development of the asci. The tips of these hyphae become free and regenerate new branches that develop into an epithecium. Ascogonial development was not discussed. Luttrell (1973) confirmed the mode of centrum development proposed by these workers and noted that ascogonia arose in the stroma. Luttrell believed this to be the Patellariaceae type centrum. Bellemère (1971) appears to accept the idea of Muthappa (1967) that paraphysoidal filaments precede ascal formation but stated that true paraphyses, which he called secondary paraphyses, are also generated later by the parathecium of the ascocarp. Kamat and Anahosur (1973) commented on the development of three members of the Patellariaceae, species of Lecanidion, Tryblidaria, and Rhytidhysterium, without adding substantially to what had been reported previously in this family.

Other species of Rhytidhysterium and related genera of the Hysteriales have been studied in respect to centrum development (Anahosur, 1974; Bellemère, 1967, 1971; Kamat and Anahosur, 1973; Muthappa, 1970; Pyrozynski and Reid, 1966; and Reid and Pirozynski, 1966). These studies showed variations in the centrum development even within the boundaries of a single genus (e.g., Rhytidhysterium).

From previous studies of R. rufulum, it can be concluded that a) different criteria have been used to distinguish taxa, b) there is some

confusion about the exact pattern of centrum development, and c) no cytological investigation of ascus development has been made. The purpose of this study, therefore, is to reevaluate the criteria presently used in the taxonomy of Rhytidhysterium and of the family Patellariaceae, to reexamine a number of aspects of centrum development, to study the cytological aspects of ascus development and ascosporogenesis, and to compare ultrastructural features of the Rhytidhysterium ascus with those of other loculoascomycetes.

Materials and Methods

Collection and Isolation

Several collections of R. rufulum were made in the orchard of the Fruit Crops Department on the University of Florida campus. Collections were made during the fall and winter of 1975 and the spring of 1976. These consisted of dead or partially dead branches of the rough lemon, Citrus jambhiri Lush., and sweet orange, Citrus sinensis (L.) Osbeck. Similar collections were made in March, 1976, at the Agricultural Research and Education Center at Lake Alfred, Florida. Samples of these collections are deposited in the Mycological Herbarium of the Florida State Museum (FLAS F 50732 and F 50733).

Isolations were made in the laboratory by allowing the ascocarps to shoot ascospores onto the surface of 2% water agar in petri dishes. This was done by attaching moistened ascocarps to the inner surface of the covers with petroleum jelly so that the hymenium faced downward. Multisporic cultures were initiated by transferring portions of the agar containing germinating spores to other culture media. Monosporic

cultures were established from single, ungerminated ascospores which were transferred with a glass micro-needle to other media. The following media were employed: potato dextrose agar (PDA), corn meal agar (CMA), Sabouraud-dextrose-agar (SAB), Emerson YpSs Agar (YPS), mycological agar (MYC), and orange stem agar (OSA). These were all Difco dehydrated media with the exception of OSA, which was prepared as follows: 100 g mature orange stems were boiled for 30 min; the resulting decoction was completed to one liter with distilled water to which was added 20 g agar. On occasion, 2% glucose or 2% microcrystalline cellulose (Sigmacell, type 50) was added to OSA.

Subcultures of R. rufulum were also made on autoclaved orange and lupine stems prepared as follows: 7 cm pieces of orange or lupine stem were placed in a test tube to which sufficient distilled water was added to cover the lower third of the stems. Cotton was packed around the base so that stem pieces would stand firmly upright. The material was autoclaved at 20 lb pressure for 30 min. After cooling, stems were inoculated with small blocks of agar containing mycelium. All cultures were kept at room temperature (24-26 C) and at 28 C.

Development and Cytology

Ascocarp initials at various stages of development were removed from cultures on autoclaved orange stems. These unfixed initials were then sectioned longitudinally in a cryostat. Five to eight micron sections were obtained and then mounted in lactophenol with cotton blue (Stevens, 1974). Sections were photographed and observed under both bright field and phase contrast illumination. Other ascocarp initials were fixed in

3:1 absolute ethanol : glacial acetic acid (v:v) for 30 min previous to being sectioned in the cryostat. Sections were fixed to microscope slides with a mixture of Haupt's adhesive and formalin (Bissing, 1974). Preparations were then hydrolyzed in 60% H_3PO_4 and stained with Giemsa according to Fernandez and Duran (1975).

Squash mounts of ascocarps containing asci and ascospores at various stages of development were stained for nuclei with the HCl-Giemsa procedure of Furtado (1968a), with the following modification: pre-treating fragments of ascocarps with a mixture of 1:1 10% KOH : 30% H_2O_2 (v:v). The material was boiled in this solution for about 15 min or until the fructification appeared bleached. This treatment removed the dark pigmentation from the ascospore walls. Material was transferred directly to the fixative for Giemsa staining.

Electron Microscopic Procedures

Fresh ascocarps were fixed for 2 hr at room temperature in a buffered (0.2 M sodium cacodylate, pH 7.2) solution of 2.0% glutaraldehyde and 2.0% paraformaldehyde (Karnovsky, 1965). The fixative was washed out with 1:1 buffer : distilled water (v:v) for 1 hr and then postfixed in buffered 1.0% osmium tetroxide overnight at 4 C. The material was then rinsed several times with the water : buffer mixture and passed through an ethanol graded dehydration series at 25% increments. During the dehydration process the material was stained with 2% uranyl acetate for 2 hr at room temperature or overnight at 4C while in 75% ethanol. Subsequently, the ascocarps were washed twice with acetone over a period of 2 hr. They were then infiltrated with a graded acetone-plastic series

and embedded in 100% plastic. The plastic used was a low-viscosity resin mixture formulated by Spurr (1969) (10.0 g ERL 4206, 6 g DER 736, 26.0 g nonenylsuccinic anhydride (NSA), and 0.4 g dimethylamino ethanol (DMAE)).

The material was left for 4 hr at each change during the entire acetone-plastic series. At the last change the material was infiltrated for 2 hr and then polymerized for 1 day in a 60 C oven.

Thick sections (1μ) were cut with glass knives to secure the proper orientation of the specimens. They were stained with crystal violet and observed under phase contrast. All ultrathin secions were cut on a Sorvall MT-2 ultramicrotome with a diamond knife and placed on single-hole formvar-coated grids. They were then normally poststained in 0.5% uranyl acetate for 15 min and in lead citrate for 5 min. A silver methenamine stain was also usually used to demarcate structures rich in polysaccharides (Martino and Zamboni, 1967). Freshly cut sections were oxidized in 2% periodic acid for 10 min at room temperature, washed, and then put in a solution of silver methenamine for 30 min to 1 hr. The staining mixture consisted of 10 ml of stock solution A (100 ml of 3% hexamethylenetetramine with 5 ml of 5% silver nitrate) and 0.8 ml of stock solution B (Borax, 5% in distilled water). The mixture was then centrifuged at 2000 rpm for 30 min while the sections were being cut. The ultrathin sections were viewed with an Hitachi-HU 11E electron microscope.

Spermatization

Masses of spermatia were aseptically transferred with a transfer needle to a test tube containing 5 ml sterile distilled water. Drops of the spermatial suspension were placed on a microscope slide and

examined with the microscope to determine the concentration of spermatia. With a sterile pipette, one drop of spermatial suspension was placed on the top of several ascocarp initials growing on sterile orange stems in culture tubes.

Results

Cultural Studies

Essentially all mature ascocarps collected from the field freely liberated spores when attached to petri dish lids above a medium. Ascospores of R. rufulum germinated readily on 2% water agar. Before germination the spores swell and become lighter in color (Fig. 47). Upon germination each ascospore usually gave rise to a single germ tube which originated either from the polar or median cells (Fig. 48). Occasionally, more than one germ tube per ascospore was observed. Mycelium derived from single ascospores grew rapidly in all culture media tested. The resulting colonies were dark olivaceous-brown and felty in appearance. Spermogonia (Figs. 1, 2) were produced after 15 to 20 days on corn meal agar and on autoclaved orange and lupine stems incubated at room conditions. Ascocarps were produced erratically, and only on autoclaved stems (Fig. 8). Spermogonia originated below the surface of the substrate, but through stromatic growth became erect as coral-like outgrowths (Fig. 1). A slimy mass of spermatia formed on the top of the spermogonia but soon became dry (Fig. 2, arrows). In cross section the spermogonia appeared as flask-shaped pycnidia, each with a sessile apical ostiole and a round to pyriform, single locule, 60-240 x 60-132 μ, enclosed in pseudoparenchymatous walls (Fig. 3). The inner surface of

the locule is lined with spermatiophores (Fig. 4). The spermatiophores are ampuliform, stout, unicellular, hyaline, 6.6-10.8 x 3.0-3.6 μ (Figs. 5-6). Spermatia are globose, smooth, unicellular, hyaline to slightly olivaceous, 2.4-3.6 (-4.2) μ in diameter, and formed endogenously at the tips of spermatiophores (Figs. 5-6). The spermatia could not be induced to germinate. Application of spermatia suspension to the ascocarp initials did not enhance apothecia production.

On autoclaved stems, the ascocarp initials appear within 25-30 days after inoculation as globose, dark, stromata (Fig. 10). They originate beneath the periderm but soon become erumpent. At this stage they appear in cross section as pulvinate stromatic pellets with outer layers of large, dark, thick-walled cells, and an inner core of hyaline, small, thin-walled cells. With continued growth they became elongate, cylindrical and covered with hyphae (Figs. 12-23). On many occasions, development of the ascocarp initials stopped at this stage, while other initials, however, continued to develop and form ascocarps at the extremity of long stipes (Figs. 8, 21). The ascocarps produced on autoclaved orange stems were identical to those formed in nature (Fig. 9), except for the long stipes and sometimes smaller size. Asci, ascospores and paraphyses were similar in the ascocarps produced *in vitro* and *in vivo*.

Development

At an early stage of ascocarp development, when the ascocarp initials are still globose (Fig. 10), no sporophytic or hymenial differentiation can be observed. Cross-sections of such initials show that their internal structure is an undifferentiated mass of thin-walled, hyaline cells which do not stain deeply (Fig. 11). When the initials begin to elongate

(Figs. 13, 14), signs of internal differentiation may be noted. Several deeply staining hyphae appear with their tips oriented toward the center of the initial (Fig. 15). A future zone of dehiscence is delineated through the invagination of a narrow area at the top of the initial (Figs. 14, 16, 17). A pre-hymenial region differentiates just below the apical invagination of the initial. This region results from the formation of a paraphysoidal plectenchyma composed of vertically oriented hyphae attached at both extremities (Figs. 16, 17, 18). At the same time, deeply staining elements (ascogonia?) appear further down the stroma (Figs. 16, 17, arrows). Some of these elements are shown at high magnification (Figs. 22, 23). A closer examination of the paraphysoidal hyphae show that their branching is directed toward both the base (Fig. 19, arrow) and the apex of the initial (Fig. 20, arrow). From the ascogonia (Figs. 22, 23), ascogenous hyphae irradiate in a fan-shaped fashion toward the pre-hymenial region (Figs. 24, 25). At high magnification the ascogenous hyphae appear slightly distended at their apices and appear to contain only a single nucleus (Figs. 26, 27). Croziers were observed just below the base of the pre-hymenial region (Fig. 28). Young asci resulting from croziers appear to push their way toward the paraphysoidal plectenchyma (Figs. 29, 30). The mature asci that developed among the paraphysoids were cylindrical, bitunicate, 168-198 x 12-26.5 μ, with a prominent ocular chamber and contained eight, dark brown, ellipsoid ascospores, 26-31.5 x 9.5-11 μ in size, which were at first two-celled, then four-celled. The hymenium is at first covered by a stromatic layer (Fig. 31) which later begins to gelatinize and separate from the asci-gerous layer (Fig. 32). This covering stromatic layer eventually crumbles

and exposes a reddish, flat disc. This disc is composed of an entangled mass of paraphysoidal branches imbedded in a gelatinous matrix. This matrix contains a red pigment which colors the hymenium. This pigment dissolves in KOH and stains blue in iodine, causing the paraphysoid apices and ascal tips to appear iodine positive.

Ascus Development and Cytology

Development of the asci begins with the formation of croziers. Atypical crozier formation was sometimes noted (Fig. 28). The nuclear condition of the crozier, and karyogamy, could not be followed in the material examined. Chromosomes and chromatin in all subsequent stages stained well with the HCl Giemsa, but centrioles, nucleoli, and nuclear membranes were not visible. After karyogamy, the young ascus containing the post-fusion nucleus increases to about 3/4 the size of the mature ascus. Uninucleate, diploid asci were abundant (Fig. 34, the nucleus of the ascus to the right is at early prophase I). Results of the first meiotic division are evident in which the two nuclei (Fig. 35, right) appear to be undergoing metaphase II. The second division of meiosis results in a serial arrangement of four nuclei (Fig. 36). The two upper nuclei appear to be at early metaphase III, although this cannot be established with certainty because a typical spindle is not apparent. Seven chromosomes can be counted in the uppermost nucleus. After the second division of meiosis is completed, a mitotic division occurs which yields eight nuclei (Fig. 37). Seven chromosomes can be counted again in the seventh nucleus from the top. A second mitotic division occurs which results in a 16-nucleate ascus (Fig. 38). At this stage ascosporogenesis begins and cleavage furrows appear which delimitate the ascospores

(Fig. 38, arrows). Thin, hyaline walls replace the cleavage furrows and the ascospores become two-celled (Fig. 39). Additional mitotic divisions and spore septation occur which lead to four-celled ascospores with uninucleate or binucleate cells (Figs. 40, 41). It is possible that the uninucleate cells undergo another mitotic division so as to make all ascospore cells equally binucleate. This, however, could not be demonstrated. The chromosome number during the second meiotic division and first mitotic division is tentatively determined as n = 7. Nuclear condition of paraphyses is variable, mostly uninucleate (Fig. 42), but occasionally binucleate. A more variable number of nuclei was observed in vegetative hyphae, usually from one to four per cell (Figs. 43, 44, 45), but ranging as high as seven.

Ultrastructure of the Ascus Wall

Silver methenamine, used to demonstrate polysaccharides, stained ascospore walls much more intensely than the ascus walls. In young asci the stain concentrated more in the outer wall of the tip (Fig. 49). A layering of the wall could not be observed at this stage. The stain was more evenly distributed in mature asci, although a more intense zone was notable at the very apex (Fig. 50), as well as a distinct layering of the ascus wall. Beneath the light, thin exoascus (Fig. 50) three layers are visible: layer a) thin, dense; layer b) thicker, less opaque; and layer c) very thick, dense and striated. After the ascus completes its maturation and approaches the time of spore ejection, there is a thinning of the apical dome, which becomes densely stained, and a return to a non-layered condition of the walls (Fig. 51). In sections stained with uranyl acetate and lead citrate, no layering of the wall could be detected.

However, a banded pattern of microfibrils was clearly observed in the endoascal wall (Fig. 52). These divergent bands of microfibrils appear to be situated in an area which corresponds to the thick endoascus (Fig. 50).

Discussion

The mode of ascospore germination in R. rufulum is very similar to that reported by Muthappa (1970) for R. clavispora (Pk.) Berl. Results of the present cultural studies agree with those presented by Shear (1933) and Voorhees (1939) except that a Diplodia-like macropycnidial stage was not found by me, and no apothecia developed on CMA or PDA. The fact that ascocarps were obtained only on autoclaved stems of orange and lupine suggests that some chemical and/or physical requirements needed by the fungus are provided by these substrates and not by the media tested. The erratic way in which ascocarps were produced indicates that some factors such as humidity, light, nutrients, or pH were not adequately controlled in the present experiments. Voorhees (1939) obtained ascocarps of R. rufulum from single ascospore cultures grown on PDA and CMA in test tubes and petri dishes. I obtained similar results using autoclaved orange and lupine stems. Voorhees also observed spermagonia in some of his cultures, but concluded that spermatization was not necessary for the production of ascocarps. This conclusion was confirmed in the present study in which ascocarp production was not enhanced by placing spermatial suspensions on developing initials. Perhaps spermatization, however, occurred very early in the development of the ascocarp, at a stage before the initials appear. In view of the present results, we must agree with the conclusion of Voorhees that R. rufulum is self-fertile

and hermaphroditic. Since all nuclei of a particular ascospore are the product of one of the tetrads, the species is homothallic. Pycnidia and pycnidiospores of an unidentified Diplodia were observed in nature on orange stems also bearing R. rufulum. However, no cultural evidence that these two species are forms of a single organism could be obtained, despite the large number of isolations made. It is likely that the Diplodia-like stage obtained by Shear (1933) and Voorhees (1939) from R. rufulum was the result of contamination.

The development of ascocarps reveals that paraphysoids (sensu Wehmeyer, 1975) are involved in the delimitation of a pre-hymenial area during centrum formation. At a later stage, asci were seen pushing through the paraphysoids. The paraphysoids were termed "vertical hyphae" by Seshadri and Muthappa (1974) and "bulbous pseudoparaphyses" by Kamat and Anahosur (1973). It is felt that such new terms are not necessary or even desirable since the term paraphysoid, redefined by Wehmeyer (1975), accurately describes the sterile filaments formed in the young hymenium. These paraphysoids do not form as the pseudoparaphyses reported by Luttrell (1953) in Glonium stellatum. Thus, R. rufulum does not have a Pleospora-type as indicated by Seshadri and Muthappa (1974). The opinion of Luttrell (1973) that the type of centrum development in R. rufulum is unique and characteristic of the Patellariaceae seems to be well founded. The mode of formation of the disc was found to be as previously described by Seshadri and Muthappa (1974) and Luttrell (1973). It is identical to that of Eutryblidiella sabina (Pirozynski and Reid, 1966) and R. clavispora (Muthappa, 1970). The interpretation of the origin of the epithecium by these authors was confirmed by present observations. True paraphyses

were not found during this study, although Bellemère (1971) suggested that they were probably present. According to Bellemère, the ascocarp of R. rufulum is structurally analogous to the apothecia of Godronia fuliginosa (Fr.) Seaver. As in R. rufulum, the development of apothecia of G. fuliginosa starts with the formation of a paraphysoidal plectenchyma. However, judging from Bellemère (1967), true paraphyses are formed in G. fuliginosa and the origin of the epithecium is different in both species. In view of the evidence accumulated thus far, and despite the superficial similarities of R. rufulum to the Ascohymeniales (Nannfeldt, 1932), it is better to assign this fungus to the Ascoloculares (Nannfeldt, 1932) or loculoascomycetes (Luttrell, 1951, 1955).

Cytological studies of the asci in the Patellariaceae have been greatly neglected. In the only detailed cytological account in the genus Rhytidhysterium, Muthappa (1970) reported the chromosome number to be n = 4 in R. clavispora. The chromosome number in R. rufulum seems to be either 6 or 7. Differences in the chromosome number among species of the same genus have been reported in Hypoxylon (Rogers, 1969a) and Xylaria (Rogers, 1969b). Since R. rufulum and R. clavispora differ in the mode of centrum formation, this further difference in chromosome numbers may not be totally unexpected. In R. clavispora, ascosporogenesis begins when the ascus reaches the eight-nucleate stage (Muthappa, 1970). In R. rufulum, however, ascospore delimitation begins only after the ascus becomes 16-nucleate. During meiotic and mitotic divisions in the ascus and ascospores, a nucleolus and a nuclear membrane were not clearly observed. The absence of the nucleolus is probably not a consequence of staining with Giemsa, since Rogers and Stiers (1974) observed that

the nucleoli in the asci of Rosellinia mammiformis (Pers. ex Fr.) Ces. & de Not. and R. aquila (Fr.) de Not. stained in their HCl-Giemsa procedure. Additional work is needed to determine whether the nuclear membranes and nucleoli disappear during divisions of nuclei in R. rufulum. Loss of the nucleolus and nuclear membrane was reported by Stephen (1971) in Lophodermium pinastri (Schrad.) Chev. Otherwise, nuclear behavior within the ascus of R. rufulum does not differ significantly from that considered typical of most ascomycetes.

The ultrastructure of the ascus wall of some Patellariaceae and Hysteriaceae was investigated by Bellemère (1971). He found that the ascus wall in R. rufulum, at the level of the apical dome, was characterized by two exoascal layers and two endoascal layers. In the material presently studied, the exoascus was composed of two layers, the inner one thicker and less opaque. The endoascus is composed of three layers: the outermost, electron opaque; the middle, less opaque; and the innermost, thick and very opaque. Bellemère (1971) considered the two innermost layers as one layer, but in his Fig. 5 all five layers are depicted. An identical number of similar layers has also been observed by the author in one species of Hysterographium (unpublished data). The endoascus of R. rufulum, as previously reported by Bellemère (1971), is transversed by banded microfibrils. This was demonstrated in my material in sections stained with uranyl acetate and lead citrate. The banded pattern of microfibrils observed in R. rufulum is similar to that reported in Limacinula theae Syd. & Butl. (Reynolds, 1971) and Myriangium duriaei Mont. & Berk. (Bezerra, unpublished). Reynolds (1971) offers an elegant explanation of the role of the banded microfibrils in the expansion of the

ascus wall and ascospore expulsion. It is possible that these banded microfibrils are exclusive of the bitunicate ascus capable of the jack-in-the-box type of dehiscence. The endoascus in a bitunicate ascus is distinct from the ectoascus in two ways: a) it is deposited only when the ascus mother cell has fully expanded, after the exoascus is formed (Reynolds, 1971); b) it has a different arrangement of the microfibrils. The structure of the endoascus seems to change with the development of the ascus. This may be due in part to a process of polymerization and depolymerization of its constituents (Furtado and Olive, 1970). The changes observed in the layering pattern of the ascus wall of R. rufulum stained with silver methenamine may be a reflection of this phenomenon.

From the analysis of known characteristics of Rhytidhysterium, it can be concluded that the relationships of this genus are with the family Patellariaceae. Rhytidhysterium is closely related to Eutryblidiella and Tryblidaria. Studies done on representatives of these genera showed that they have a common type of centrum (Pirozynski and Reid, 1966; Muthappa, 1967; Kamat and Anahosur, 1973; Anahosur, 1974; Seshadri and Muthappa, 1974). Lecanidion may also be related, but the presence of true paraphyses in this genus may indicate affinities elsewhere. This is also true of Tryblidiella clavispora which also has true paraphyses (Muthappa, 1970). When more developmental studies are carried out with other species, it may be found that those Patellariaceae with large, cylindrical to long-clavate asci, containing mostly phragmospores or dictyospores, will form a very natural group. On the other hand, it is likely that the didymospored Patellariaceae with short, clavate asci, like Buellia and Melaspilea, will have to be placed elsewhere. The

affinities between the Patellariaceae and Hysteriaceae, judging from developmental patterns and hymenial configurations, may be more distant than is presently accepted. It is questionable whether these two families should be placed in the same order. A decision to reposition these families, however, will await further research.

Chapter I

Figures 1-7. The spermogonial stage of Rhytidhysterium rufulum.

1. Coraloid spermogonia on CMA. X40.
2. Masses of spermatia (arrows) formed on the top of spermogonia on CMA. X40.
3. Longitudinal section through a spermogonium. X40.
4. Spermogonial wall in section, lined with spermatiophores. X1,000.

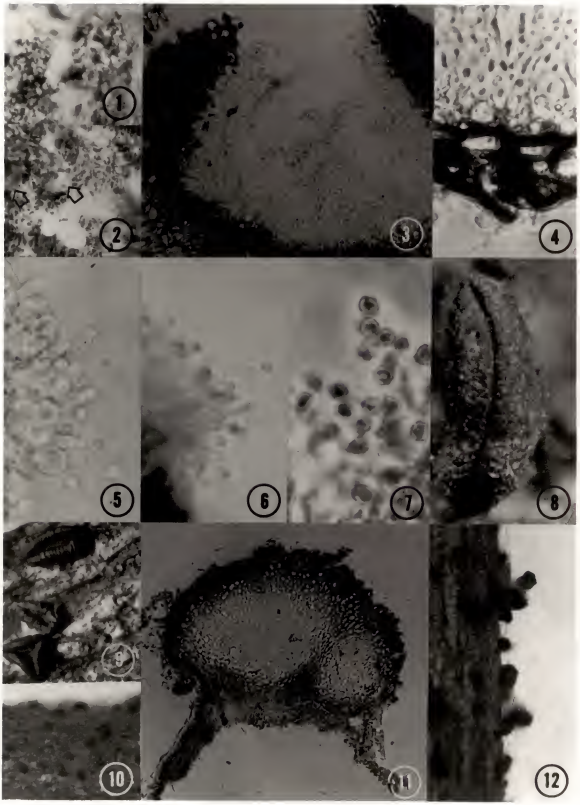
5-7. Spermatia produced on spermatiophores. X1,000.

Figures 8-9. The ascocarp of Rhytidhysterium rufulum.

8. Ascocarp formed on autoclaved orange stem. X40.
9. Ascocarps on orange stem in nature. X20.

Figures 10-12. Developmental stages of Rhytidhysterium rufulum in vitro.

10. Round ascocarp initials. X20.
11. Longitudinal section through an ascocarp initial at the stage shown in Fig. 10. X200.
12. Cylindrical ascocarp initials. X20.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Figures 13-21. Developmental stages of Rhytidhysterium rufulum.

13. Close-up of an elongate ascocarp initial. X40.

14. Longitudinal section of an ascocarp initial at the stage shown in Fig. 13. X100.

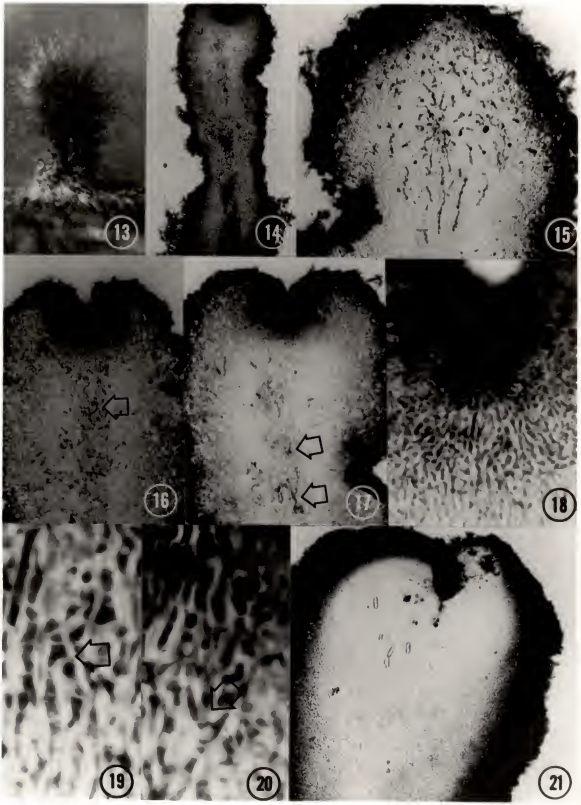
15. Longitudinal section of an ascocarp initial showing beginning of centrum differentiation. X200.

16-17. Longitudinal section of an ascocarp initial showing pre-hymenial layer and ascogonia (arrows). X200.

18. Paraphysoids which form the pre-hymenial layer. X400.

19-20. Detail of paraphysoidal branching (arrows). X1,000.

21. Longitudinal section of a mature ascocarp. X100.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Figures 22-33. Developmental stages of Rhytidhysterium rufulum.

22-23. Ascogonia. X1,000.

24-25. Ascogenous hyphae. X400.

26-27. Uninucleate ascogenous hyphae. X1,000.

28. Crozier below paraphysogenous plectenchyma. X1,000.

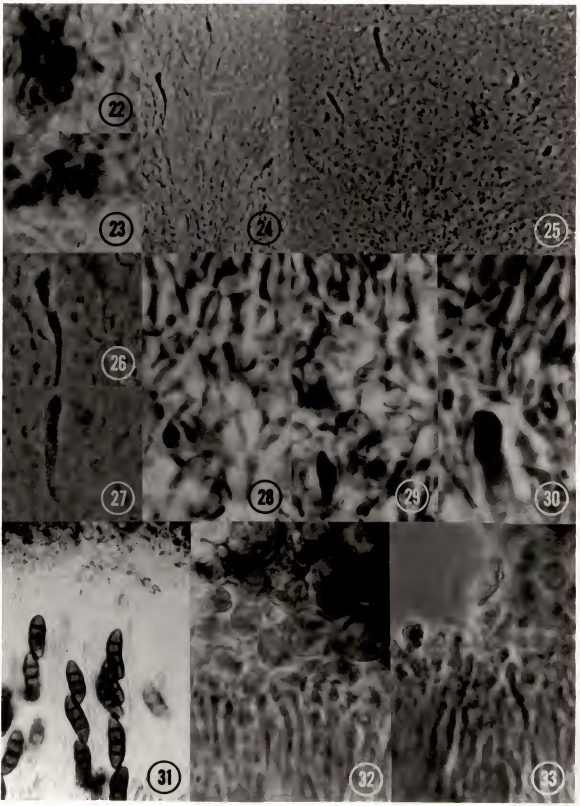
29. Young ascus below paraphysogenous plectenchyma. X1,000.

30. Maturing ascus pushing through paraphysogenous plectenchyma. X1,000.

31. Mature asci and ascospores among paraphysoids. X400.

32. Tips of paraphysoids covered by an epistroma. X1,000.

33. Crumbling of epistroma. X1,000.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Figures 34-42. Cytology of asci, ascospores and paraphyses of Rhytidhysterium rufulum.

34. Two uninucleate asci. X1,000.

35. Two binucleate asci. X1,000.

36. Four-nucleate ascus. X1,000.

37. Eight-nucleate ascus. X1,000.

38. A 16-nucleate ascus. X1,000.

39. Two-celled, young ascospore with two uninucleate cells. X1,000.

40. Four-celled ascospore with uninucleate and binucleate cells. X1,000.

41. Ascus tip with two four-celled ascospores with uninucleate cells. X1,000.

42. Predominantly uninucleate paraphysoidal tips. X1,000.

Figures 43-45. Nuclear condition of hyphae of Rhytidhysterium rufulum.

43. A trinucleate hyphal cell. X1,000.

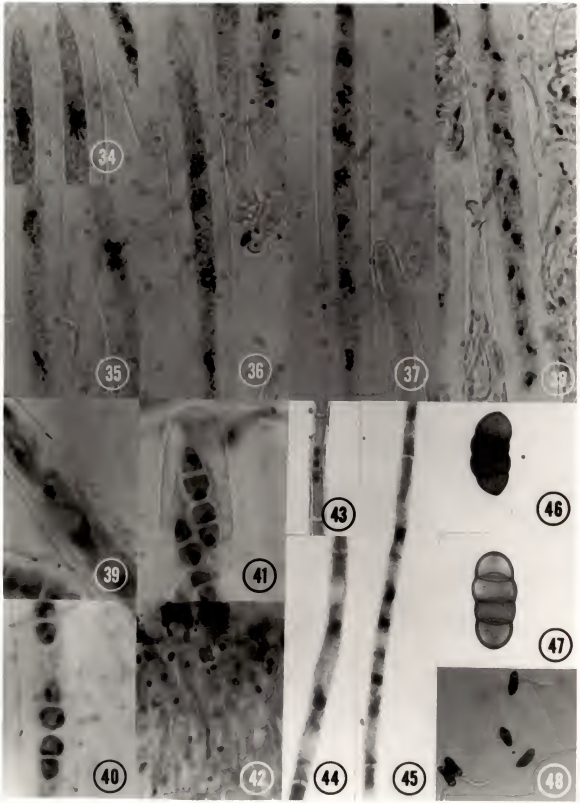
44. A binucleate hyphal cell. X1,000.

45. A four-nucleate hyphal cell. X1,000.

Figures 46-48. Pre-germination and germinating ascospores of Rhytidhysterium rufulum.

46-47. Swollen pre-germinating ascospores. X1,000.

48. Germinating ascospores. X200.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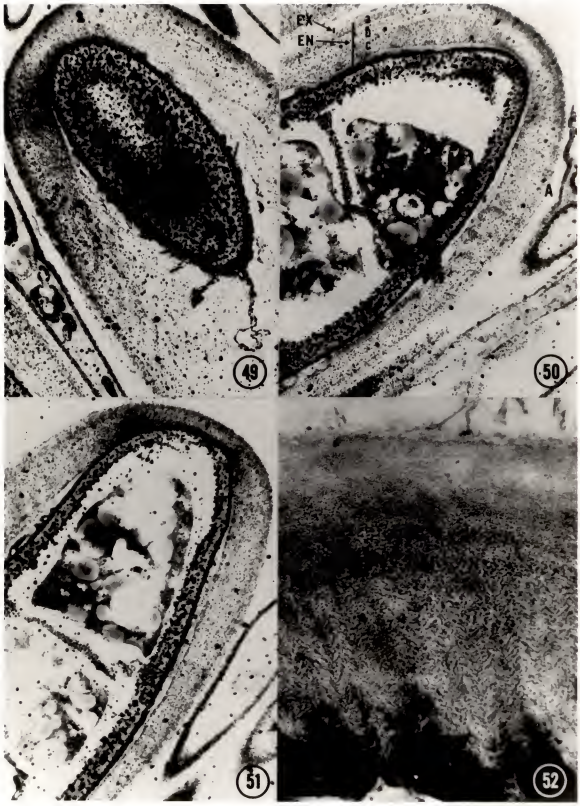
Figures 49-52. Ultrastructure of the ascus of Rhytidhysterium rufulum.

49. Young ascal tip and ascospore. X8,700.

50. Mature ascal tip and ascospores. X8,700.

51. Ascal tip approaching time of ascospore ejection. X8,700.

52. Ascal tip showing the banded pattern of microfibrils in one endoascus. X36,000.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CHAPTER II
A NEW SPECIES OF *Tremella* PARASITIC ON *Rhytidhysterium rufulum*

During the course of my studies on the development and cytology of *Rhytidhysterium rufulum* (Spreng.) Speg., ascocarps of this fungus collected in Gainesville, Florida, were commonly found colonized by a tremellaceous basidiomycete. The latter consisted of pulvinate, gelatinous, brown fructifications. These consisted of clamped hyphae which produced either obliquely or transversely septate basidia bearing subglobose, hyaline basidiospores typical of the genus *Tremella*. A search of the literature revealed the following facts: a) no Tremellales are reported on *Rhytidhysterium* or any other member of the Patellariaceae; b) three species of ascomycetes are described as mycoparasites of species of *Rhytidhysterium*; and c) a number of species of the genus *Tremella* are reported as mycoparasites of pyrenomycetes and basidiomycetes.

The following species of ascomycetes are parasitic on *R. rufulum*: 1) *Mollisiella ilicincola* (Berk. & Br.) Massee (=*Cenangium ravenellii* Sacc.) reported by Seaver (1939) and Voorhees (1939) on both *R. rufulum* and *R. fuscum* (Ell. & Ev.) Bezerra & Kimbr., comb. nov.[1]; 2) *Pleospora rhytidhysterii* Pet. on *R. rufulum* (Petrak, 1962); and 3) *Leptosphaerulina mycophaga* Batista & Bezerra on *R. fuscum* (Batista and Bezerra, 1966). The following species of *Tremella* are described as mycoparasites:

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[1] *Tryblidium rufulum* var. *fuscum* Ell. & Ev. is congeneric with *R. rufulum*, although it is a distinct species (Voorhees, 1939). The proper combination of the former taxon into *Rhytidhysterium* was never made. Therefore, this new combination is proposed: *Rhytidhysterium fuscum* (Ell. & Ev.) Bezerra & Kimbr.

1) T. encephala Pers. ex Pers. on Stereum sanguinolentum Fr. (Reid, 1970); 2) T. episphaerica Rick on Diatrype sp. (Rick, 1958); 3) T. exigua Desm. on pyrenomycetes (Donk, 1966); 4) T. indecorata Sommerf. on pyrenomycetes (Reid, 1970; Torkelsen, 1968); 5) T. globospora Reid on Diaporthe eres Nke. (Brough, 1974; Reid, 1970); 6) T. mycetophiloides Kobay. on Aleurodiscus sp., (Kobayasi, 1939); 7) T. mycophaga Martin on Aleurodiscus amorphus (Pers.) Rabenh. (Martin, 1940); 8) T. obscura (Olive) Christ. on Dacrymyces spp. and other Dacrymycetales (Olive, 1946, 1948; Christiansen, 1959; McNabb, 1964); 9) T. parasitica Fr. on Clavariaceae species (Saccardo, 1888); 10) T. polyporina Reid on Tyromyces lacteus (Fr.) Murr. (Reid, 1970); 11) T. pyrenophila Trav. & Migl. on Valsaria insitiva (Fr.) Ces. & de Not. (Donk, 1966); and 12) T. simplex Jacks. & Mart. on Aleurodiscus amorphus (Reid, 1970).

Among the Auriculariales and Tulasnellales, mycoparasitic species have been described which belong to the genera Helicogloea (Olive, 1951), Mycogloea (Olive, 1958), Platygloea (Olive, 1946, 1954, 1958; Rick, 1933; Burdsall, 1974), and Tulasnella (Olive, 1944, 1953). The characterization of the mycoparasitism of the species listed above is based mostly on circumstantial evidence, such as the presence of the "mycoparasite" on or in the fructification of its host. No experimental work has been attempted to prove the pathogenicity of tremellaceous species. The associations between mycoparasitic species of Tremella and their basidiomycetous hosts have been regarded as symbiosis by Dangeard (1895) and as teratological aberrations by Ramsbottom (1933). Since it became established that the association involved two different fungal species, and that the host fungus was adversely affected, both theories have been

refuted. Although mycoparasitism is presently accepted, Barnett and Binder (1973), who reviewed the subject, failed to mention Tremella among their list of mycoparasites. Substantial evidence of parasitism by species of Tremella, such as haustorial formation or antagonistic cultural responses, is limited. Bandoni (1961) illustrated haustoria of Tremella encephala Pers. attached to a hypha of Stereum sanguinolentum (Alb. & Swein.) Fr. Olive (1946a) made a similar observation of an unidentified species of Tremella on Dacryomyces minor Pk. Koske (1972) grew mixed cultures of Tremella uliginosa Karst. and its host, a sterile deuteromycete, and noted that in the absence of the host no fructifications were formed and only limited mycelial growth occurred.

The occurrence of a species of Tremella on R. rufulum in Florida provided the author an opportunity to study the interaction between these two organisms. This fungal combination represented an exceptionally good working system because both fungi could be cultured in the laboratory. Research was initiated for the following purposes: a) to identify the species of Tremella found on Rhytidhysterium, b) to study the mycelial interaction and reproduce mycoparasitism in vitro, c) to observe interactions at both light and electron microscope levels, and d) to study the cytological development of basidia and basidiospores.

Materials and Methods

Collection and Isolation

Several collections of T. rhytidhysterii were made during the fall of 1975 and spring of 1976 in the orchard of the Fruit Crops Department on the campus of the University of Florida, Gainesville, Florida. These

consisted of parasitized ascocarps of R. rufulum on branches of rough lemon, Citrus jambhiri Lush. Specimens from the New York Botanical Garden (NY) and the University of Florida Herbarium (FLAS) were also examined for the presence of the parasite.

Isolations were made in the laboratory by transferring single probasidia with a glass micro-needle to several different media in petri dishes. The following media were used in the isolation and growth experiments: malt and yeast extract - soytone agar (MYS, Brough, 1974), conjugation medium (CJM, Brough, 1974), potato dextrose agar (PDA), corn meal agar (CMA), Sabouraud - dextrose agar (SAB), Emerson YpSs agar (YPS), Czapek Dox agar (CZ), and mycological agar (MYC). Orange stem agar (OSA) and autoclaved orange stems (Chapter I) were occasionally used. These were all Difco dehydrated media except MYS and CJM.

Mycelial Interaction

Tremella rhytidhysterii was grown on a thin layer of CZ and half-strength CMA for 15 to 20 days in petri dishes. A growth of R. rufulum was established 3 or 4 cm away on the same dish. As soon as hyphae of the two fungi made contact, a rectangle about 18 x 36 mm was cut from the culture in the contact area. This block was removed and aseptically transferred to a sterile slide, covered with a sterile 22 x 40 mm cover slip, and maintained in a moist chamber. Slide cultures of this type were observed and photographed over a period of several days without staining.

Inoculations of R. rufulum ascocarps with Tremella was accomplished by using living ascocarps produced in the laboratory on autoclaved orange stems in test tubes (Chapter I). Fragments of Tremella mycelium

were aseptically transferred to the hymenial areas of mature ascocarps. The yeast phase was inoculated in a like manner.

Cytology

Vegetative hyphae were stained using the H_3PO_4 - Giemsa method of Fernandez and Duran (1975). Slide cultures bearing contact hyphae were fixed with 3:1 absolute ethanol : glacial acetic acid (v:v) for 30 min. After fixation, the culture was rinsed briefly in 90%, 70%, and 50% ethanol and two changes of tap water respectively. The material was then inverted on microscope slides coated with a mixture of Haupt's adhesive and formalin (Bissing, 1974) and dried on a slide warmer at 40-45 C. Preparations were then placed overnight in 60% H_3PO_4 at room temperature to hydrolyze and remove the agar. Slides with attached mycelium were rinsed first in tap water, then in Sorensen's buffer at pH 6.5. Nuclei were stained 30 min in 1 part Giemsa stock solution (Ward and Ciurysek, 1961) and 25 parts phosphate buffer. Differentiation and dehydration were done with an acetone : xylene series (Duncan and Galbraith, 1973), and the specimens were then mounted in Harleco synthetic resin.

Basidiospores and basidia of T. rhytidhysterii showed good nuclear staining using the HCl - Giemsa procedure of Furtado (1968). Fragments of the basidiocarp were stained within 3-11 hr, washed, and mounted in buffer. Good nuclear staining was also obtained for squash mounts stained with lactophenol cotton blue (Stevens, 1974) and with lactofuchsin. Observations were made under bright field and phase contrast illumination.

Electron Microscopic Observarions

Fresh basidiocarps, collected in the field or produced in the laboratory, were fixed in Karnovsky's fixative (1969) and embedded in Spurr (1969) resin. Staining was done with periodic acid - silver methenamine (Martino and Zamboni, 1967) and also with uranyl acetate and lead citrate. Details of these procedures are presented in Chapter I.

Results

Taxonomy

The tremellaceous fungus encountered as a mycoparasite of Rhytidhysterium rufulum was studied closely on its natural host and in culture. Basidiospores were found to be typical of the genus Tremella, but septation of the basidia was atypical of that described for most species. However, descriptions have been made of some species of Tremella which have similar, atypical, two-celled basidia. These are: T. encephaliformis Willd. (Olive, 1946a), T. polyporina Reid (Koske, 1972), T. mycophaga Martin var. obscura Olive (Olive, 1946b), and T. simplex Jacks. and Martin (Torkelsen, 1967). Since the species under study cannot be accommodated by any of the currently described taxa, the following new species is described:

Tremella rhytidhysterii Bezerra and Kimbrough, sp. nov.
Figs. 1-15.

Fructificationes sessiles, pulvinatae, firme-gelatinosae, 0.2-1.0 mm diam, brunneae, rugosae, leviter farinosae, coalescentes; hyphae conspicue nodosae, 1.5-4.0 μ diam; basidia elliptica vel ovata, 2-cellularia, hyalina vel lutescentia, 15-30 x 10-13 μ, sterigmata dua,

3.0-4.5 μ diam producentia; basidiosporae subglobosae vel ample ovatae, hyalinae, levae, apiculatae, 7.8-11.4 x 7.8-10.8 μ.

Fructifications parasitic, 0.2-1.0 mm diam, pulvinate, brown, with a surface smooth at first, then somewhat rugose or sulcate; confluent, almost entirely covering the host ascocarp; shrinking slightly on drying, becoming hard, nodulated, dark brown bodies; not hollow in section; hyphae thin-walled, irregular, 1.5-4.0 μ in diam, clamp connections abundant, conspicuous; probasidia mostly elliptical to broadly fusiform, sometimes subglobose to ovate, two-celled, with a transverse or oblique septum, 15-30 x 10-13 μ, each with a conspicuous basal clamp through which proliferation may occur; paraphyses lacking; protosterigmata extremely variable in length, 3.0-4.5 μ diam, basidiospores subglobose to ovate, apiculate, continuous, hyaline, 7.8-11.4 x 7.8-10.8 μ, thin-walled, germinating by repetition or by germ tube formation; conidia lacking.

Name: Referring to the host genus.

Specimens examined: UNITED STATES: Bezerra, Univ. of Florida, Gainesville, FL, Dec., 1975 (FLAS F 50734, TYPE); Voorhees, Gainesville, FL, Oct. 10, 1937 (NY); H. W. R., Houston, TX, April 7, 1869 (NY); Earle, Auburn, AL, April 8, 1900 (NY); W. B. Cooke, Mt. Repose, OH, Nov. 10, 1935 (NY). CENTRAL AMERICA: Griggs and Underwood, Bayamon, PR, June 14, 1901 - July 22, 1901 (NY); Ekman, prov. de Santiago, Valle del Cibao, Dom. Rep., Feb. 27, 1930 (NY). SOUTH AMERICA: Baker, Santa Marta, Colombia, Nov., 1898 (NY). ASIA: Reinking, Los Banos, Philippines, Nov. 4, 1919 (NY); S. Y. Cheo, Kweichow Province, China, Sept. 21, 1931 (NY).

The fructifications of T. rhytidhysterii are restricted to the hymenial surface of the host and are not found growing elsewhere (Fig. 3). The hyphae penetrate deeply into the hymenium of the host (Fig. 4) and are also observed around stromatic host cells. The parasite appears to be widespread, and it is likely that when herbarium specimens of Rhytidhysterium are examined closely the geographic and host range of T. rhytidhysterii will be greatly extended.

Cultural Studies

Cultures derived from single probasidia were either filamentous or yeast-like, or a combination of both stages. The filamentous form was found to consist of dikaryotic hyphae with clamp connections. Nutrient-rich culture media, such as PDA or SAB, stimulated the production of a yeast phase. Likewise, mycelial cultures were easily maintained on nutrient-weak media, such as Czapek Solution agar or half-strength CMA. By streaking yeast-like isolates, dikaryotization was induced, but not consistently. Thus, dikaryotization was not followed at the microscopic level. Primary hyphae sometimes originated from basidiospore germination, but conjugation was not detected between primary hyphae, between yeast cells, or between yeast cells and hyphae. Dedikaryotization, resulting in yeast formation from the filamentous phase, was frequently observed. Such hyphae become very granulose at first (Fig. 31), and then appear moniliform, at which stage yeast cells develop (Figs. 33, 34). The daughter cells have the same granulose consistency as the parent hyphal cells (Fig. 32). The process of yeast cell formation is apparently more complex than simple budding or blastospore formation. Temperature did not appear to affect dikaryotization or dedikaryotization, but did

affect growth. Cultures grown at 28 C grew very poorly, in contrast to good growth observed for those grown at 15 C and 25 C. Colonies in the yeast phase were whitish to creamy in color and smooth and slimy. Mycelial colonies were white, raised or flattened, smooth, and cottony to fluffy. Nutrients, on the other hand, appear to exert an influence on both dikaryotization and dedikaryotization. Isolations from single probasidia on rich culture media invariably resulted in yeast-like or mixed colonies. Identical results were noted when mycelial isolates were subcultured on nutrient-rich media.

Mycoparasitic Mycelial Interaction

Ascocarps of Rhytidhysterium rufulum produced in the laboratory and inoculated with mycelium of T. rhytidhysterii became infected and after three to four days developed gelatinous basidiocarps on the hymenial surface. Basidiocarps of Tremella formed in vitro were identical to those observed in the field (Figs. 1, 2). The mycoparasitic hyphae could be observed colonizing the hymenial elements (Fig. 4) and the stromatic cells of the host (Fig. 5). Ascocarps of the host did not become infected when inoculated with the yeast phase of the parasite. Even though a number of media were tested, including autoclaved orange stems, the parasite developed basidiocarps only on the host.

When mycelia of the two fungi were grown together (Fig. 37), the host was not inhibited and the parasite did not sporulate. As a rule, the mycelium of Tremella was completely overgrown by Rhytidhysterium. Strikingly, however, in the presence of the host hyphae, there was a great increase in the number of haustorial branches from the hyphae of Tremella (Figs. 37-43). In the absence of the host these haustorial

branches were seen to approach and make contact with host hyphae (Figs. 37-43), but no penetration was observed. In one instance, signs of cytoplasmic deterioration of the host cell in contact with haustorial cells was observed (Fig. 39). With the electron microscope, hyphae of the parasite were noted growing between and laterally adpressed to host cells (Figs. 44-46). A haustorial branch was also observed with its tip squeezed between two adjacent walls of the septum of a host cell (Figs. 46, 47). The septal pore of T. rhytidhysterii is an atypical dolipore with a fragmented parenthesome or pore cap consisting of U-shaped vesicles (Figs. 48, 49). These vesicles are formed by membrane layers and appear to be connected by microfilaments to the center of the dolipore channel.

Cytology

The secondary hyphae of T. rhytidhysterii are binucleate and clamped (Fig. 28). When in the presence of host mycelia, each clamp connection of these hyphae develops into a haustorial branch which contains a single basal nucleus (Fig. 29). Yeast-like cells are uninucleate and result from monosporic isolations or from the dedikaryotization of secondary mycelium (Figs. 34, 35).

Probasidia arise from binucleate mother cells (Fig. 16) which undergo karyogamy (Fig. 17), increase in size (Figs. 18, 19), and support meiosis. From the first meiotic division arise two nuclei which are separated by a septum, resulting in a metabasidium with two uninucleate cells (Fig. 20). The second meiotic division is not synchronous and may start in either the upper (Fig. 21) or the lower (Fig. 22) cell of the metabasidium. At the end of the second meiotic division, additional

septa are not formed and the metabasidium now consists of two binucleate cells (Fig. 23). Each metabasidial cell gives rise to one protosterigma through which the two nuclei are seen to migrate (Figs. 25-27). Uninucleate basidiospores develop (Fig. 24), indicating that one nucleus disintegrates, or does not migrate into the newly developing spore.

Fine Structure of Homokaryotic Cells

In thin sections, the capsular material is shown to be composed of filamentous projections of electron-opaque material (Figs. 50, 51). These projections branch and anastomose at their bases, forming a reticulum which is particularly noticeable at the connection between two cells (Fig. 50). The cell walls are bilayered with an outer, lighter zone, and an inner, more opaque zone (Fig. 50). The plasma membrane appears crenulated and invaginated (Fig. 51). The cytoplasmic matrix is not uniform throughout the cells, but has an irregular, electron-transparent peripheral zone of glycogen (Fig. 51). The ribosomes are evenly distributed in each cell but are excluded from the flocculent portions of the cytoplasm. Mitochondria are elongate or circular in section and irregularly distributed in the cell (Fig. 50). Vacuoles were not observed, but lipid bodies and vesicles are frequent. A single nucleus with a conspicuous nucleolus is visible in each cell (Fig. 51). The nuclei are irregular or circular in cross section. Inclusion bodies and concentric membrane complexes are often seen in the cytoplasm (Fig. 51).

Discussion

Tremella rhytidhysterii has a unique combination of characters that make it an easily-identifiable species. It is the only species of

Tremella found on this host, and its peculiar basidial shape and septation distinguish it from other species. In identifying mycoparasitic species, attention has been given to 1) the color, size, position in the host, and solidness or hollowness of the basidiocarps; 2) the shape and septation of basidia; and 3) the presence or absence of clamps and conidia. Brough (1974) believes that color variation in Tremella basidiocarps may result from genetic differences and maturation, and suggests that the use of color to separate species should be applied with caution.

The ability to grow species of Tremella on defined media has provided mycologists with the opportunity to perform studies on conjugation and dikaryotization (Bandoni, 1963; Kobayasi and Tubaki, 1965; Brough, 1974). Dedikaryotization was observed by Brough (1974) in T. globospora. However, the present study appears to be the first documentation of the morphological sequence of dedikaryotization in Tremella. Further work on the cytochemical transformation and ultrastructural changes during dedikaryotization is strongly recommended. The role of nutrients in the regulation of dikaryotization and dedikaryotization is poorly understood. Conjugation hormones (erogens) have been implicated in the process of dikaryotization in T. mesenterica Fr. (Reid, 1974). Dikaryotization of yeast-like cells induced by the presence of host hyphae was also reported by Bandoni (1961) in T. encephala Pers.

The subject of mycoparasitism was reviewed by Barnett (1964), Boosalis (1964), and Barnett and Binder (1973). Tremella rhytidhysterii may be classified as a biotrophic (balanced) contact parasite (Barnett and Binder, 1973). This category of parasitism includes those forms which do not produce internal haustoria or hyphae and do little harm to

their host, unlike the group of necrotrophic or destructive parasites. Only five species of biotrophic contact mycoparasites, all hyphomycetes, were recognized by Barnett and Binder (1973). Gams (1974) added a monotypic genus Tympanosporium (Moniliaceae) to this group. Basidiomycetes, until now, were not recognized among the biotrophic contact mycoparasites; Tremella is the first basidiomycete to acquire this status.

Studies on the mycelial interaction between biotrophic contact mycoparasites and their hosts were conducted mainly with the hyphomycetous representatives (Shigo, 1960; Whaley and Barnett, 1963; and Gams, 1974). Bandoni (1961) grew cultures of T. encephala Pers. and Stereum sanguinolentum (Alb. & Schw. ex Fr.) Fr. together, and noted that haustorial branches were produced on the hyphae of Tremella. The observations made by Bandoni, although incomplete, were the first carried out in culture. Olive (1946a, 1946b) made pioneering observations on haustorial formation by a parasitic species of Tremella in nature. Brough (1974) described the formation of haustorial branches by T. globospora Reid in vitro. Koske (1972), studying T. uliginosa Karst. in culture, noted the formation of gripping basal cells instead of haustorial branches. The results presently reported indicate that haustorial production by T. rhytidhysterii is greatly enhanced by the presence of the host hyphae. However, they can sometimes develop, in reduced numbers, in the absence of the host. Brough (1974) also observed the formation of haustorial branches in T. globospora growing alone in culture.

The ultrastructure of the host-parasite relations between Allomyces and Rozella was investigated by Held (1972). De la Cruz and Hubbell (1975) also studied the fine structure of the association between an

unidentified basidiomycete and Macrophomina phaseolina (Tassi) Goid. Similar studies involving biotrophic contact mycoparasites and their hosts are lacking. The intimate relation between hyphae and haustorial branches of T. rhytidhysterii and its host is clearly demonstrated in the electron microscopic observations carried out in this work. No penetration was detected, confirming the observations made with the light microscope. The fact that the haustorial branches of the parasite do not actually penetrate the host hyphae is a condition of its biotrophic contact mycoparasitic nature. Calderone and Barnett (1972) postulated that hyphal contact, even without penetration, is essential for the establishment of a nutritional relationship between the parasite and its host.

The nuclear condition of T. rhytidhysterii hyphae in the presence of the host is striking. The existence of a nucleus at the base of haustorial branches has not been previously reported. The significance of this finding is not clear, but it may become important in the future. It may be that the haustorial branches will become homokaryotic. Should this be the case, haustorial branch formation may provide the fungus with another means of reverting to the homokaryotic phase.

The cytology of basidial development in Tremella has been studied by Whelden (1934) and Furtado (1968b). The accounts given by these authors on the nuclear phenomena taking place in the basidium do not differ significantly from my observations. T. rhytidhysterii seems to be unique in the nonseptation of the basidium after the second meiotic division and in the migration of two nuclei through one protosterigma. The fact that the basidiospore is uninucleate implies that one of the nuclei did not migrate into the basidiospore or degenerated before reaching that point. Degeneration of post-meiotic nuclei is known to occur

among the Dacrymycetales (Bessey, 1961), although it has not been recorded in Tremella.

My observation of the ultrastructure of the homokaryotic cells of T. rhytidhysterii agrees with that reported by Bandoni and Bisalputra (1971) for T. mesenterica (Gray) Pers., with the exception of the ultrastructure of the capsule. The capsular material of T. rhytidhysterii is unique for its filamentous configuration. The occurrence of capsules around the wall of vegetative cells is common among the yeasts, particularly the Cryptococcaceae. Lodder and Kreger-Van Rij (1952) describe the capsule of Cryptococcus as containing a starch-like polysaccharide. A metagenetic relation between the Tremellales and yeast has been suggested by some authors (Lodder and Kreger-Van Rij, 1952). Kobayasi and Tubaki (1965) studied the cultural and physiological characters of members of the heterobasidiomycetes, hoping to clarify their relationship with the anascosporogenous yeasts. They found that the yeast phase of T. fuciformis Berk. corresponds very closely to Cryptococcus neoformans (Sanf.) Vuill. Kwong-Chung (1975) induced conjugation between strains of C. neoformans and obtained a perfect stage which she named Filobasidiella neoformans. It would be very interesting to determine whether species of Cryptococcus and Filobasidiella possess a capsular fine structure similar to that of T. rhytidhysterii.

The septum between cells of T. rhytidhysterii has a peculiar ultrastructure. A dolipore is present and the pore cap consists of U-shaped vesicles. A similar pore structure was described by Kreger-Van Rij and Veenhuis (1971) in Filobasidium capsuligenum (Fell et al.) Rodr. de Miranda. Moore and Kreger-Van Rij (1972) made a more detailed study of the septal structure in that species and in F. floriforme Olive. Olive

(1968) placed Filobasidium in the order Ustilaginales on the basis of basidial septation and parasitic habit. In view of presently available information, the taxonomic position of Filobasidium needs reconsideration. Primarily, the septal structure in this genus points not to the Ustilaginales but to the Tremellales. Moore (1972) placed great emphasis and phylogenetic importance on septal structures in characterizing his new fungal class Ustomycota, a group in which dolipores and parenthosomes are lacking. Other features now known to exist in the Tremellales and also found in Filobasidium are: the mycoparasitic habit, the formation of haustorial branches in the presence of a fungal host, and the induction of dikaryotization in the mycoparasite by hyphae of the host. In T. uliginosa (Koske, 1972) the probasidia which are subtended by fertile hyphae do not form basidiospores but function themselves as "spores". Could the basidium and basidiospores in Filobasidium be analogous, respectively, to the fertile hypha and probasidium of T. uliginosa? The correct answer to this question may be obtained through cytological and genetic studies which can determine whether karyogamy and meiosis occur in the "basidia" of Filobasidium and whether the "basidiospores" genetically behave as such. The fact that conidiophores and conidia occur in Tremella has to be taken into consideration in any attempt to interpret the reproductive structures of Filobasidium and Filobasidiella. More evidence substantiating the idea of a phylogenetic relationship among these three genera was provided by Kwong-Chung (unpublished data) who recently found that Filobasidiella has a dolipore septum similar to that of Tremella and Filobasidium.

Chapter II

Figures 1-6. *Tremella rhytidhysterii* on ascocarps of *Rhytidhysterium rufulum*.

1-2. Fructifications of *T. rhytidhysterii* covering the ascocarp of *R. rufulum*. X20.

3. Longitudinal section of an ascocarp bearing a fructification of *T. rhytidhysterii*. X100.

4. Hyphae of *T. rhytidhysterii* in the hymenium of *R. rufulum*. X400.

5. Hypha of *T. rhytidhysterii* surrounding a stromatal cell of *R. rufulum*. X1,000.

6. Probasidia of *T. rhytidhysterii* magnified from Fig. 3. X400.

Figures 7-15. Morphology of basidia, basidiospores, and germination of basidiospores of *Tremella rhytidhysterii*.

7-8. Metabasidia at early stages of protosterigmata formation. X1,000.

9. Metabasidium with protosterigmata and attached basidiospores. X1,000.

10-11. Metabasidia and protosterigmata. X1,000.

12. Basidiospore attached to protosterigma. X1,000.

13. Basidiospores. X1,000.

14. Basidiospore germinating by a germ tube. X1,000.

15. Basidiospore germinating by repetition. X1,000.

Figures 16-22. Cytology of the basidium of *Tremella rhytidhysterii*.

16. Binucleate basidial primordium. X1,000.

17. Basidial primordium after karyogamy. X1,000.

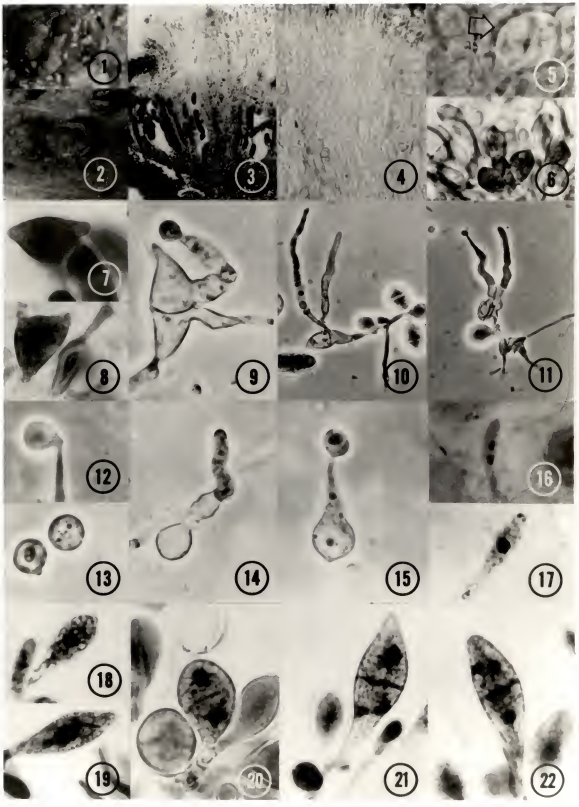
18. Diploid basidial primordium increasing in size. X1,000.

19. Diploid nucleus undergoing division in the basidial primordium. X1,000.

20. Binucleate basidium after first meiotic division. X1,000.

21. Probasidium with lower cell undergoing second meiotic division. X1,000.

22. Probasidium with binucleate upper cell after second meiotic division. X1,000.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Figures 23-29. Cytology of the basidium and hyphaeof Tremella rhytidhysterii.

23. Four-nucleate basidium. X1,000.

24. Uninucleate basidiospore. X1,000.

25-27. Nuclear migration of the nuclei through the protosterigmata. X1,000.

28. Nuclear condition of hypha without haustorial branches. X1,000.

29. Nuclear condition of hypha with haustorial branches. X1,000.

Figures 30-32. Reversion from the mycelial phase to the yeast phase in Tremella rhytidhysterii.

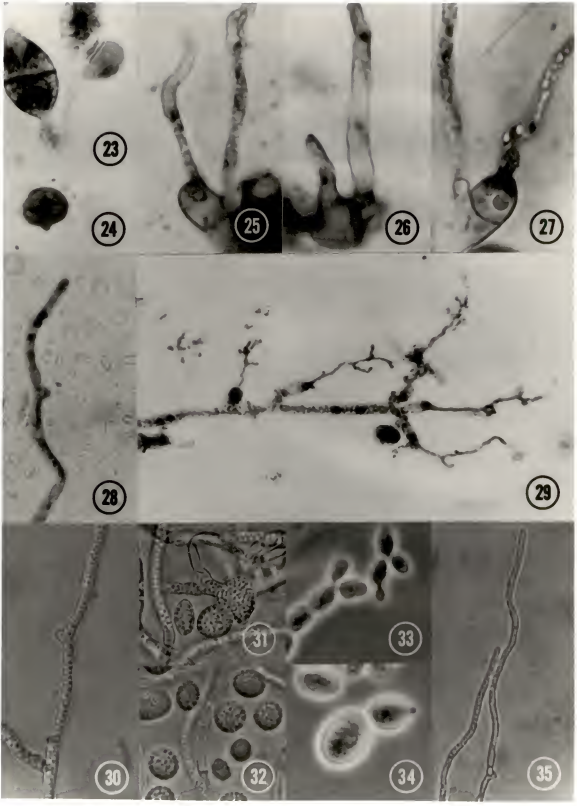
30. Hypha with granulose cytoplasm. X1,000.

31. Hypha with granulose cytoplasm giving rise to yeast cells. X1,000.

32. Yeast cells with granulose cytoplasm. X1,000.

Figures 33-34. Budding of yeast cells of Tremella rhytidhysterii. X1,000.

Figure 35. Normal hyphae of Tremella rhytidhysterii formed in the absence of Rhytidhysterium rufulum. X400.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Figures 36-45. Mycelial interaction between Tremella rhytidhysterii and Rhytidhysterium rufulum.

36. Mycelia of T. rhytidhysterii and R. rufulum coming into contact. X200.

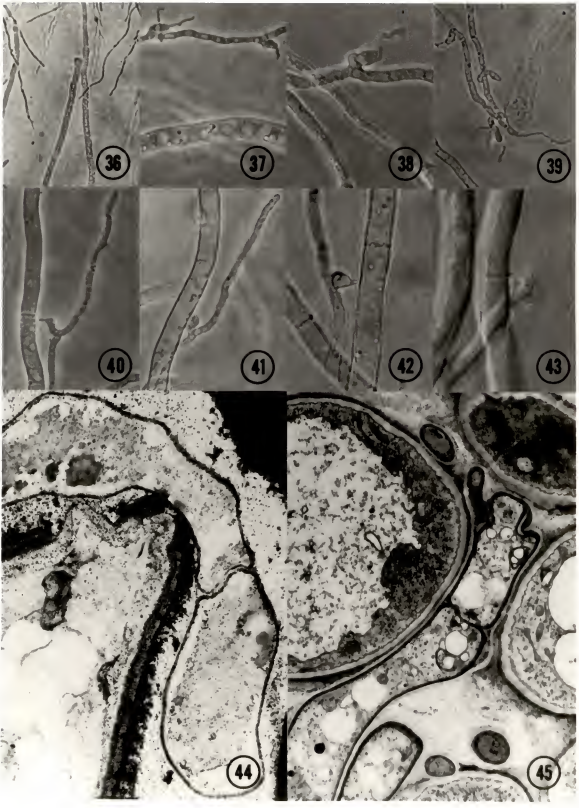
37-38. Haustorial branches developed by T. rhytidhysterii hyphae in presence of R. rufulum hyphae. X1,000.

39. Hypha of R. rufulum showing cytoplasm alteration after contact with T. rhytidhysterii hyphae. X1,000.

40-43. Haustorial branches of T. rhytidhysterii contacting the hyphae of R. rufulum. X1,000.

44. Hypha of T. rhytidhysterii surrounding a stromatal cell of R. rufulum in section. X17,000.

45. Hypha of T. rhytidhysterii positioned between sectioned paraphysoids of R. rufulum. X9,200.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Figures 46-47. Mycelial interaction between Tremella rhytidhysterii and Rhytidhysterium rufulum.

46. Haustorial branches of T. rhytidhysterii contacting hyphae of R. rufulum. X15,300

47. Detail of Fig. 46 showing tip of a haustorial branch positioned between two adjacent walls of the septum of R. rufulum. X43,700.

Figures 48-49. Ultrastructure of the dolipore septum of Tremella rhytidhysterii.

48. Part of clamp connection with two dolipore septa. X42,000.

49. Dolipore septum showing the pore channel and U-shaped vesicles of the pore cap. X127,200.


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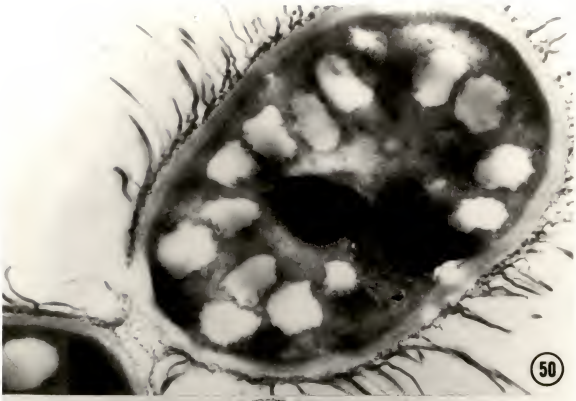
Figures 50-51. Ultrastructure of yeast-like cells of Tremella rhytidhysterii.

50. Budding cells with abundant filamentous capsular material. X13,050.

51. Non-budding cell with little capsular material (CW, cell wall; PZ, less electron-opaque zone in cytoplasm; M, mitochondrium; IB, inclusion body; L, lipid body; MC membrane complex; N, nucleus; NU, nucleolus). X17,450.



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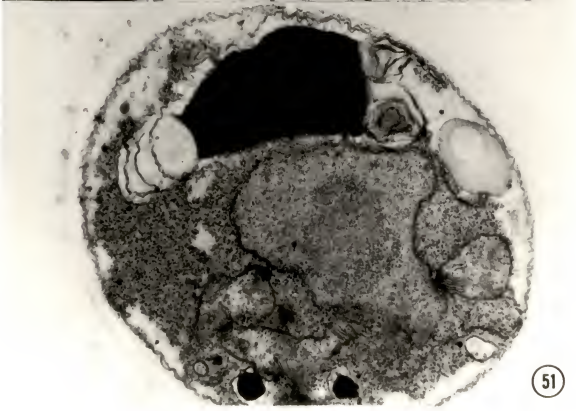

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CHAPTER III
HOST-FUNGUS RELATIONSHIP OF *Rhytidhysterium rufulum* ON *Citrus*

The literature pertaining to *Rhytidhysterium rufulum* (Spreng.) Speg., an apothecioid loculoascomycete (Patellariaceae), reveals that this fungus has a broad host range (U. S. Dep. Agric., 1960; Muthappa, 1967; Shear, 1933; Voorhees, 1939). It has been regarded as pathogenic to citrus plants by several authors (U. S. Dep. Agric., 1960; Bhatnagar, and Prasad, 1966; Deighton, 1936; Subba Rao, 1937). Moreau and Moreau (1951) believed that *R. rufulum* was one of the most pathogenic fungi attacking coffee; it causes tracheomycosis. Subba Rao (1937) reported the occurrence of *R. rufulum* on orange trees previously infected by *Corticium salmonicolor* Berk. and Br. *Rhytidhysterium rufulum* was thought to be the agent of twig blight of lime (U. S. Dep. Agric., 1960; Knorr, 1973). In India, Bhatnagar and Prasad (1966) believed the same species caused canker and dieback of lime, and in that country, *R. rufulum* was reported on branches of several citrus species by Sydow and Butler (1911), Ghosh and Dutta (1962) and Dwivedi (1962).

Information on the pathogenicity of *R. rufulum*, based on experimental evidence, is nonexistent in the literature. Bhatnagar and Prasad (1966) speculated that this fungus may function as a wound parasite.

Since *R. rufulum* occurs on species of *Citrus* in Florida, a state where the citrus industry is particularly important, this investigation

was undertaken with the following purposes: a) to establish whether or not R. rufulum is pathogenic on Citrus in Florida, b) to investigate the fungal agents of any discovered disease, and c) to study the mechanism of host penetration and colonization by these agents if a disease was encountered.

Materials and Methods

Collection and Isolation

Several collections of R. rufulum were made during the fall and winter of 1975 and spring of 1976 in the orchard of the Fruit Crops Department on the University of Florida campus, Gainesville, Florida. These specimens consisted of dead or partially dead branches of the rough lemon, Citrus jambhiri Lush., and the sweet orange, C. sinensis (L.) Osbeck. Similar collections were made in March, 1976, at the Agricultural Research and Education Center at Lake Alfred, Florida. Portions of these collections are deposited in the Mycological Herbarium of the Florida State Museum (FLAS F 50732, and F 50733).

Isolations and subcultures were carried out using the procedures outlined previously in Chapter I of this dissertation except that Czapek Dox Agar (Difco) was used in addition to the other culture media listed.

Inoculations in the Laboratory

Living branches of the rough lemon tree (C. jambhiri) were cut into 7 cm pieces and disinfected by 1) soaking in 10% chlorox for 10 min, followed by three distilled water washes or 2) dipping in alcohol and flaming twice. Two series of moist chambers were prepared by

autoclaving petri dishes lined with moist filter paper and subsequently placing into each dish three pieces of disinfected stem from one or the other treatments above.

An ascospore suspension was obtained as follows: test tubes containing autoclaved citrus stems, on which ascocarps of R. rufulum had developed, were examined. Those tubes in which a considerable amount of ascospores had been shot onto the walls were flooded with sterile distilled water and shaken. Three drops of the ascospore suspension were aseptically deposited onto each stem piece in the moist chambers. Two series of ten plates were thus prepared, one series containing living disinfected stems, the other containing flamed stems. For each series, two control plates were made in which only sterile water was applied to the stems. The moist chambers were kept on the laboratory benches at room temperature (ca 22-23 C) and examined at 12 hr intervals over a five-day period. Examination was made under a stereomicroscope at 40 X without opening the petri dishes. Some of the stems were removed from the moist chamber when there was an indication of ascospore germination. A thin layer of the periderm of these stems was removed with a razor blade, placed on microscope slides in water, observed and photographed at higher magnifications through a compound microscope. The material was then placed in a cryostat, sectioned, stained with lactophenol cotton blue (Stevens, 1974), and subsequently observed at higher magnifications for hyphal penetration and colonization.

Inoculations for Greenhouse and Growth Chambers

Inoculations were made on potted rough lemon plants treated or grown as follows: 1) starved (grown in washed sand), 2) non-starved

(grown in standard potting mix), 3) cold-treated non-starved, 4) heat-treated non-starved. Heat treatment was given to only a few branches of each plant by applying an alcohol lamp flame for ca 30-40 sec, in order to kill the branches without charring them. Cold treatment was applied to the entire plant. Leaf-freezing points (LFP) of three randomly-selected, fully-expanded mature leaves were averaged to give the LFP for each plant (Wiltbank and Rouse, 1973) (18 F in these tests). Plants were placed for 1 hr in a walk-in freeze chamber in which the temperature had been adjusted to 18 F. After treatment the plants were transported to a greenhouse. After 3-4 days all leaves wilted, but the stems remained alive and later started to form new shoots. Inoculations were made after the leaves had wilted.

The inoculum used for treatments one to three above was prepared as follows: five day old cultures of R. rufulum on CMA were suspended for 2 min in a Waring blender, and the mycelial suspension was applied to the plants with a sprayer. Control plants were inoculated with sterile CMA without the fungus, prepared as above. The inoculum used in treatment four was prepared as follows: R. rufulum was grown in 125 ml Erlenmeyer flasks (ca 50 ml CZ broth per flask) on a shaker. After six days the mycelium was decanted, washed several times with sterile distilled water, suspended in 500 ml distilled water, and mixed in a blender for 2 min. The resulting mycelial suspension was applied to the plants with a sprayer. Control plants were inoculated with sterile distilled water. In all treatments, each plant was covered with a plastic bag after inoculation and placed in a plastic tray containing water.

Both cold-treated and heat-treated plants were additionally inoculated with Diplodia natalensis Evans to determine whether a relationship exists between R. rufulum and D. natalensis. Mycelial suspensions of D. natalensis were applied as described above for R. rufulum. Table 1 summarizes the experiments performed on rough lemon plants.

Results

Field Observations

Ascocarps of R. rufulum were seen in the field on stems of Citrus jambhiri which had been damaged by Diaporthe citri Wolf and Gleosporium limetticola Clausen. On C. sinensis, R. rufulum was observed on stems which apparently were killed by D. citri. Both D. citri and G. limetticola were isolated in culture and identified in the laboratory. Signs of frost damage were also noted on the trees on which R. rufulum was found; there are some indications that R. rufulum also developed on branches which were previously frost damaged. The population of Rhytidhysterium observed in Gainesville, Florida, was heavily parasitized by Tremella rhytidhysterii Bezerra & Kimbr. (Chapter II). Apparently T. rhytidhysterii serves to keep the population of the fungal host under control. Rhytidhysterium rufulum was never observed associated with living branches in the field.

Inoculations in the Laboratory

Ascospores of R. rufulum germinated within 12 hr on both living and heat-killed C. jambhiri stems (Fig. 1). On living stems, contamination with other fungal species was minimal or nonexistent. On heat-killed stems, however, the amount of fungal contamination was

Table 1. Experiments performed on potted C. jambhiri plants after R. rufulum inoculation.

Treatment	Host	Number of treated plants	Number of control plants	Growth conditions	Temperature
1	starved	4	2	growth chamber	23 C
2	non-starved	8	2	greenhouse	27±3 C
3	non-starved, cold-treated	10	2	greenhouse	27±3 C
4	non-starved, heat-treated	5	2	laboratory	ca 28-30 C

considerable. Germ tubes produced by the ascospores, usually one to two per ascospore, did not direct themselves toward stomata or lenticels. Some germ tubes were seen passing beside a stoma without penetrating (Fig. 4). Penetration of the fungal hyphae into the cortex of the stems was observed in both living and heat-killed stems (Figs. 5, 6). The site and mode of hyphal penetration were not determined. In the host tissues the hyphae were seen growing intercellularly (Figs. 5, 6) and intracellularly (Fig. 7). The fungus was not recovered from the inoculated living stems. Reisolation from inoculated heat-killed stems was not attempted because of the presence of contaminants.

Inoculations in the Greenhouse

Results of treatments one and two were negative: no signs of the fungus or symptoms of infection were observed for two months after inoculations. Plants subjected to cold treatment showed fungal growth within 30 days after inoculation, but only on parts which had been damaged by cold. Extensive growth of fungal contaminants was noted on both inoculated plants and control plants (Fig. 8, control on right). On the inoculated plants, R. rufulum developed despite contamination (Fig. 9, young stroma on branch at right). The fungus was easily reisolated from these plants. No mature ascocarps or pycnidial stages were observed on the inoculated plants.

Within 30 days of inoculation, R. rufulum was observed on treated branches only of heat-treated plants (Figs. 10-13). The fungus produced ascocarp initials on the inoculated, dead branches; mature ascocarps were occasionally formed. Reisolation of the fungus was easily

accomplished. Pycnidia were never observed from these isolations, nor did they form on branches inoculated with R. rufulum. Both heat-treated and cold-treated plants inoculated with Diplodia natalensis produced pycnidia of D. natalensis within 30 days, but after a four-month observation period, no signs of R. rufulum were detected.

Discussion

Ascospores are the only agents of dissemination of R. rufulum that have been observed. No cultural evidence for the existence of a conidial stage was demonstrated in the present experiments. Diplodia natalensis was frequently found associated with R. rufulum in the field; however, plants inoculated with Diplodia exhibited no sign of the presence of R. rufulum, and likewise, plants inoculated with R. rufulum exhibited no sign of the presence of Diplodia. Since the ascospores and conidia of these two fungi are similar, even under low magnifications, it is possible that the Diplodia-like stage reported by Shear (1933) and Voorhees (1939) for R. rufulum was the result of contamination.

Field observations and inoculation experiments showed that R. rufulum is not pathogenic to citrus in Florida. The fungus is apparently unable to permanently establish itself in healthy host tissues but can become established on branches that are damaged by heat or cold. It is possible that frost-killed branches constitute a substrate for the fungus in the field. Rhytidhysterium rufulum seems to hasten the decay of branches attacked by Diaporthe citri and Gleosporium limetticola in the field. Ascospores of D. citri are

capable of initiating infection in citrus (Wolf, 1926; Ruehle and Kuntz, 1933; Knorr, 1973). Perithecia of D. citri are formed in decaying wood (Knorr, 1973). Field observations indicated that R. rufulum and D. citri compete for the niche provided by decaying branches, and it is possible that this competition causes a decrease in the ascospore production by D. citri. The influence of this competition on the epidemiology of melanose has yet to be determined. Since G. limetticola is thought to live from one season to the next in dead twigs (Knorr, 1973), it may also compete with R. rufulum for the same substrate. The implications here are the same as with D. citri. If R. rufulum has a beneficial role in the epidemiology of melanose and lime anthracnose, the mycoparasitism of T. rhytidhysterii is detrimental. This situation illustrates an instance in which the association between a mycoparasite and a saprophytic fungus may have phytopathological implications.

The results of the inoculations of C. jambhiri stems in moist chambers show that the living stem is not an appropriate substrate for R. rufulum. The growth of contaminants on the heat-killed stems in moist chambers is an indication that some other fungi were present internally in the stems. The fact that R. rufulum could not be reisolated from inoculated living stems may be due to the presence in the cortex of few hyphae at the time of the isolation trials. However, since no other fungi were isolated, it cannot be assumed that the hyphae observed in the tissue were contaminants. The limited evidence for hyphal penetration obtained in this work cannot constitute an indication that the fungus is a parasite of C. jambhiri. One must

also consider that injury inflicted by cutting the stems may have altered the metabolism of the stems, or that the fungus may have lost its viability some time after penetration.

The occurrence of contaminants on all plants of treatment three was thought to be a consequence of the culture medium contained in the inoculum used. Inoculum of treatment four was prepared so as to contain no culture medium. However, saprophytic fungi still developed on heat-killed branches of control plants. This is another indication that dead branches of orange constitute an adequate substrate for saprophytes. Rhytidhysterium rufulum proved to be a good competitor since it developed on inoculated plants of treatments three and four despite contaminants.

The inoculation experiments conducted strongly suggest that R. rufulum is not capable of becoming established on healthy tissue of C. jambhiri plants. There is still the possibility that R. rufulum is a perthotrophic fungus (Luttrell, 1974). However, no indication of fungal invasion of healthy tissue originating from infected dead tissue was noted. The fungus always behaved as a saprophytic fungus capable of invading dead or damaged branches of rough lemon.

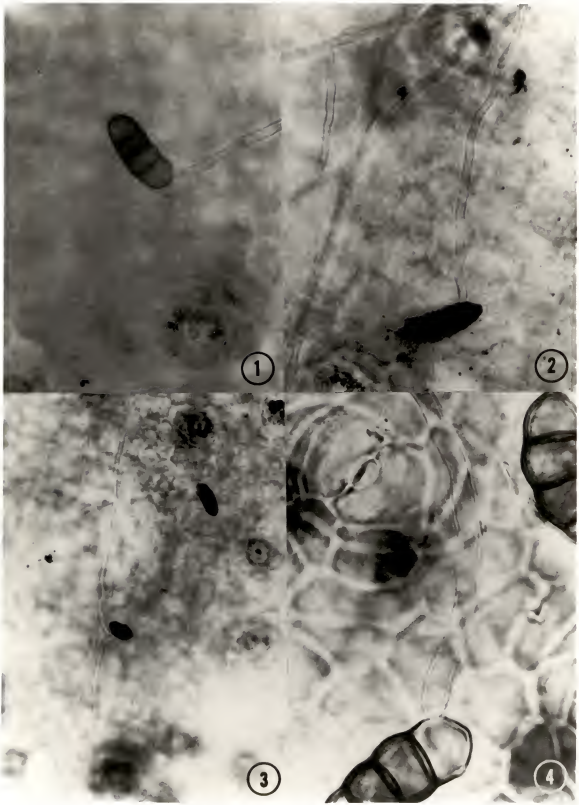
Chapter III

Figures 1-4. Ascospore germination of *Rhytidhysterium rufulum* on *Citrus jambhiri* stems in moist chambers.

1-2. Germinating ascospores with single polar germ tube. X700.

3. Two germinating ascospores and several germ tubes. X300.

4. Germinating ascospores with germ tube passing by a stoma. X1,400.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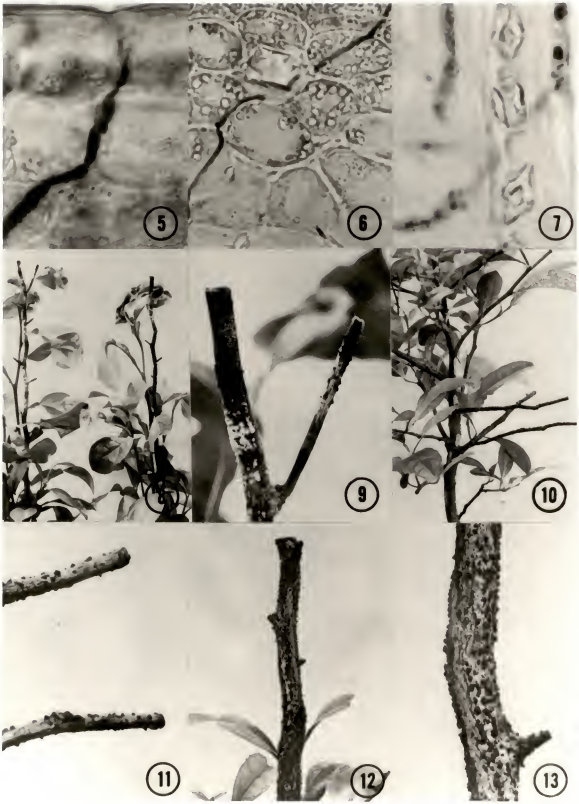
Figures 5-7. Mycelium of *Rhytidhysterium rufulum* within inoculated stems of *Citrus jambhiri*.

5. Hyphae in the cortical cells. X1,000.
6. Hyphae in the cortical cells. X400.
7. Hyphae in the parenchymatous cells of the cortex. X1,000.

Figures 8-13. *Citrus jambhiri* plants inoculated with *Rhytidhysterium rufulum*.

8. Plants subjected to cold treatment prior to inoculation. Left: plant 30 days after inoculation with *R. rufulum*. Right: control plant inoculated with sterile CMA.
9. Branches of plant shown at left in Fig. 8, showing contaminants (left branch) and young stromata of *R. rufulum* (right branch).
10. Plant with *R. rufulum* developing on heat-treated branches.
11-13. Heat-treated branches showing stromata of *R. rufulum*.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BIOGRAPHICAL SKETCH

Jose Luiz Bezerra was born January 2, 1940, in Bom conselho, Pernambuco, Brazil. In December, 1957, he received his high school degree from Colegio Salesiano in Recife, Pernambuco. He obtained a Bachelor of Science in Biology and his "Licenciatura em Biologia" from the Universidade Catolica de Pernambuco, Recife, in December, 1964, and December, 1965, respectively. From March to October, 1962, he studied mycology and plant pathology with Dr. J. A. von Arx in Baarn, Holland. In June, 1962, he joined the staff of the Instituto de Micologia in Recife, and in May, 1971, he was appointed director for a four year term. Since June, 1967, he has held a position of Lecturer in Mycology at the Universidade Federal Rural de Pernambuco. In March, 1972, he was awarded a scholarship from the U. S. Agency for International Development to study in the United States. He entered the University of Florida in March, 1972, and received his Master of Science Degree in June, 1974. From that time until the present, he has pursued his work toward the Doctor of Philosophy. He is the author or co-author of more than one hundred research articles.

Jose Luiz Bezerra is married to the former Noelia Albuquerque Trindade, and he is the father of a son, Jose Luiz, and two daughters, Marcia Maria and Katia Maria. He is a member of the Mycological Society of America, the Brazilian Society of Botany, the Brazilian Society of Plant Pathology, the Brazilian Society of Microbiology, the

Brazilian Society for the Progress of Science, and the Brazilian Foundation for the Conservation of Nature.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James W. Kimbrough, Chairman
Professor of Botany

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Norman C. Schenck, Cochairman
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Daniel A. Roberts
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Henry C. Aldrich
Professor of Botany

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dana G. Griffin, III
Associate Professor of Botany

This dissertation was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1976

Dean, College of Agriculture

Dean, Graduate School